Insulin-like Growth Factor-1 and Cardiotrophin 1 Increase Strength and Mass of Extraocular Muscle in Juvenile Chicken

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PURPOSE. Insulin-like growth factor 1 (IGF1) and cardiotrophin 1 (CT1) are known to increase the strength of extraocular muscles in adult and embryonic animals, but no information is available for the early postnatal period, when strabismus treatment in humans is most urgent. Here the authors sought to determine whether these trophic factors strengthen juvenile maturing extraocular muscles and gain insight into mechanisms of force increase.

METHODS. After two injections of IGF1, CT1, or both with different dosages in posthatch chickens, the authors quantified five parameters of the superior oblique extraocular muscle at 2 weeks of age: contractile force, muscle mass, total myofiber area, myofiber diameter, and number of proliferating satellite cells labeled by bromodeoxyuridine.

RESULTS. Treatment with IGF1, CT1, and combination of IGF1 and CT1 significantly increased contractile force by 14% to 22%. CT1 and combination treatment significantly increased muscle mass by 10% to 24%. IGF1/CT1 combination treatment did not have additive effects on strengthening muscles, compared with single-drug treatments. Myofiber area increased significantly with IGF1 and CT1 treatment in proximal, but not distal, parts of the muscle and this was due to increased fiber numbers or length (IGF1) or increased diameters of global layer myofibers (CT1). Trophic factors increased the number of proliferating (bromodeoxyuridine-labeled) satellite cells in proximal and middle segments of muscles.

CONCLUSIONS. Exogenous IGF1 and CT1 strengthen extraocular muscles during maturation. They predominantly remodel the proximal segment of juvenile extraocular muscles. This information about muscle plasticity may aid the design of pharmacologic treatment of strabismus in children during the "critical period" of oculomotor maturation. (*Invest Ophthalmol Vis Sci.* 2010;51:2479–2486) DOI:10.1167/iovs.09-4414

Strabismus is a misalignment of the visual axes or restriction of the range of gaze that can have many causes, including altered contractility of extraocular muscles (EOMs). It is a common disorder affecting 4% to 6% of the population. In infants, strabismus disrupts the development of neurovisual circuits for normal binocular vision and can cause severe deficiencies, such as amblyopia. Most strabismus treatment is surgical and limited to the manipulation of elastic muscle forces (recession and resection) and muscle pulling directions (transposition).[1] Pharmacologic manipulations of intrinsic muscle properties are promising alternatives, but these approaches, except for botulinum toxin,[2] are largely experimental.[3–5] The early postnatal or posthatch period is critical for EOM morphogenesis, force development,[6,7] and myosin heavy chain isoform transition in EOMs.[7,8] Trophic adjustments during this critical phase of development may alter the structure and function of muscles and thus may improve eye alignment in the strabismic condition.[3,5]

Insulin-like growth factors (IGFs) promote the survival and growth of cells as an anabolic hormone.[7,9] IGFs regulate the proliferation and differentiation of satellite cells to induce myogenesis and regenerate muscle.[10,11] These properties have prompted studies to strengthen underacting EOMs by local application of IGFs. Injection and sustained release of IGFs increase the strength and myofiber size of EOMs in adult rabbits.[12–14] In embryonic chickens, both exogenous and endogenous IGF1 strengthen EOMs,[15] with multiple sources of IGF1 contributing.[16] IGF1 and other cytokines, such as cardiotrophin 1 (CT1), induce the proliferation of activated satellite cells.[17–20] During muscle growth and hypertrophy, these cytokines enhance satellite cell proliferation and differentiation.[21] CT1 can increase EOM strength when applied to the orbit of embryonic chickens.[15]

We asked in this study whether IGF1 and CT1 can strengthen maturing (posthatch day 7–14) EOMs, whether the combination of IGF1 and CT1 has additive effects, and how such treatment affects muscle properties such as muscle mass, myofiber area, and myofiber diameter and number or length. This was examined after 1 week of treatment, as in previous studies.[12,14] In addition, we asked whether these trophic factors can increase the proliferation of activated satellite cells in EOMs in vivo and which parameters of muscle alteration are associated with IGF1- and CT1-induced increases of EOM strength.

MATERIALS AND METHODS

Materials

Fertilized White Leghorn chicken eggs were obtained from local suppliers and incubated at 37.0°C to 37.5°C. Date of hatching was designated posthatch day (P) 0. Approximately 200 animals were housed in brooders with controlled temperature (23°C–25°C) on a 12-hour light/12-hour dark cycle and were provided chicken feed and water ad libitum. Experimental procedures were conducted in compliance with the Policy on the Use of Animals in Neuroscience Research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local animal care committee. IGF1 and CT1 were purchased from PeproTech (Rocky Hill, NJ).

Injection Procedure for Trophic Factors

At P6 and P9, chicks were anesthetized by intramuscular injection of sodium pentobarbital (34 mg/kg; Nembutal; AmerisourceBergen Corporation, Valley Forge, PA) and chloral hydrate (150 mg/kg). A dose of either 0.5 μg or 5 μg of IGF1 or CT1 alone or of 0.5 μg IGF1 was combined with 5 μg or 0.5 μg CT1 (total volume, 10 μL) and was injected in the right dorsomedial orbit containing the superior oblique muscle. For control experiments, muscles either were not injected or were injected with an equivalent volume of sterile phosphate-buffered saline (PBS). Preparations of IGF1, CT1, and PBS were administered using disposable insulin syringes (28G1/2; Becton Dickinson, Franklin Lakes, NJ). IGF1 and CT1 were radiolabeled[21,22] and injected to verify their accumulation throughout different segments of the EOM by γ-counting. The EOMs of chicken arise from the interorbital septum (posterior wall of orbit) rather than the tendinous annulus deep at the apex of the orbit, as in mammals. Lack of a trochlea (distinct from mammalian superior oblique) facilitates the electrophysiological and morphologic analysis of the avian superior oblique muscle.

Measurements of Contractile Force and Muscle Mass

Anesthesia was induced as described and was maintained by intramuscular injection of 10 to 25 mg/kg sodium pentobarbital (Nembutal; AmerisourceBergen) every 30 minutes. The contractile force of 55 superior oblique muscles was successfully evaluated in situ with nerve and blood supply intact, as described in detail in our previous work.[23] The animal was placed on its left side, and the head was immobilized with a stereotaxic frame. The insertion of the right superior oblique muscle was exposed and tied with a 6-0 silk suture and cut from the sclera. The trochlear (IV) nerve was carefully isolated from connective tissue near its entrance to the superior oblique muscle. The suture previously attached to the global portion of the superior oblique muscle was then tied to a 1-g isometric force transducer (calibrated in the range of 100 mg–2 g; Fort 25 [World Precision Instruments, Sarasota, FL]). Parallel platinum electrodes (0.3-mm tip diameter, 1 mm between poles) contacted the portion of the trochlear nerve ~5 mm proximal to its entry into the mid belly of the superior oblique muscle. In situ muscle force of the superior oblique muscle was measured in response to trochlear (IV) nerve stimulation (15 V, 0.2-ms duration; S48 stimulator [Grass, Quincy, MA]). Optimal muscle length was determined while twitch tension was maximal isometric twitch tension. The tetanic tension was measured with train durations of 200 ms and stimulation frequencies in the range of 200 to 500 Hz (50-Hz increments). Each stimulus train was separated by a 5-second interval. Single-twitch and tetanic tensions were measured with a data acquisition system (Digidata 1322A; Axon Instruments, Union City, CA) with a sampling rate of 500 to 5000 Hz. Consistent with previous reports,[6,24] the contraction time was approximately 5.8 ms, and the range was 5.6 to 6.0 ms. The total duration of physiological stimulation experiments per animal was 10 to 12 minutes, and the duration from anesthesia to euthanatization was approximately 50 minutes. Upon the completion of contractile measurements, muscles were dissected free from their origin, trimmed of connective tissue, and blotted on filter paper, and the mass was determined on an analytical balance (BP 110S [Sartorius AG, Göttingen, Germany]).

Morphometry of Myofibers

A separate group of treated and control chickens was killed at P14 by intraperitoneal injection (Euthasol; Virbac, Forth Worth, TX) and perfused intracardially with 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). At least three chickens were used for analysis of each condition (trophic factor–treated or control). The right superior oblique muscles were dissected out and fixed in perfusion buffer for 3 hours. Muscles were cut transversely into proximal, middle, and distal segments. Muscle segments were postfixed in 1% osmium tetroxide for 1 hour and dehy-

drated in a series of graded ethanols and propylene oxide, embedded in Spurr's resin, and polymerized at 60°C overnight. The muscle was sectioned in the transverse plane. Semithin sections (1 μm) were prepared and stained with 0.7% toluidine blue. For proximal and distal parts, the sections were collected at approximately 1.5 mm from the origin and insertion of the muscle, respectively. Stained sections were examined under a microscope with the help of a counting grid (400 μm^2 for the 40× objective, 1600 μm^2 for the 20× objective, 6400 μm^2 for the 10× objective). The cross-sectional area of the entire muscle and the percentage of myofiber-occupied area were estimated by point counting.[25] The myofiber area was calculated by multiplication of the entire cross-sectional area with the percentage of myofiber-occupied area. This method for determination of the myofiber area is considered more accurate than calculating muscle volume/average fiber length, especially in EOMs.[26] Myofiber diameters were measured under the microscope with a 40× objective using counting grids (20 μm × 20 μm = 400 μm^2 for the 40× objective). We analyzed myofibers by systematic random sampling. Approximately 2% of all myofibers within the cross-sectional area were sampled. The myofiber number in cross-sections was estimated by point counting, using counting grids (40 μm × 40 μm = 1600 μm^2 for the 20× objective) for calculation of the total number of myofibers based on the total cross-sectional area and the average number of myofibers within the sampling area using an unbiased counting frame.[25] Orbital and global layers were not assessed separately because their borders in the transition zone are difficult to delineate in avian EOMs, particularly when treatment with growth factors could potentially change myofiber diameters. All quantitative data were obtained by analyzing coded specimens to avoid observer bias.

Bromodeoxyuridine Immunolabeling

The thymidine analog bromodeoxyuridine (BrdU) was used to track DNA synthesis and mitosis in superior oblique muscles of juvenile chickens. BrdU (Sigma, St. Louis, MO) in sterile phosphate buffer (10 mg/mL) was injected intraperitoneally at a dose of 50 mg/kg body weight. Chickens were injected intraperitoneally with BrdU when their orbits were injected with IGF1, CT1, or PBS (on P6 and P9). This regimen takes into account the fact that EOMs contain activated satellite cells[27] and the property of growth factors to rapidly induce proliferation.[20] The chickens were killed on P14 by intraperitoneal injection (Euthasol; Virbac) and were perfused intracardially with 4% paraformaldehyde. The superior oblique muscles were dissected out, postfixed for 3 hours, sunk in gradient sucrose solution overnight, embedded in OCT compound (Sakura Finetek Inc., Torrance, CA), and longitudinally cryosectioned at 10 μm on a cryostat (Leica, Wetzlar, Germany). For BrdU immunostaining, the sections were rinsed in PBS, incubated in 1 N HCl for 10 minutes on ice, in 2 N HCl for 10 minutes at room temperature, and for 20 minutes at 37°C, followed by neutralization in 0.2 M sodium borate buffer (pH 8.5) and PBS rinses. The sections were incubated in primary antibody (OBT0030S-monoclonal rat anti-BrdU; Gentaur, Brussels, Belgium) diluted 1:100 overnight at 4°C, rinsed in PBS; this was followed by secondary antibody (Alexa Fluor 546 goat anti-rat IgG; Invitrogen Inc., Eugene, OR). All BrdU-labeled nuclei located in or on myofibers were counted under a microscope with a 20× objective and a calibrated grid. Approximately 40 to 60 myofibers were randomly sampled for each EOM segment (proximal, middle, distal). Counts were expressed as mean ± SEM per 100 μm myofiber length, longitudinally along 1-mm sample areas for three segments (proximal, middle, distal).

Statistical Analysis

All data were analyzed statistically (SPSS 16.0 for Windows software; SPSS Inc, Chicago, IL). Data are shown as mean ± SEM. Statistical significance was evaluated at a confidence level of $P < 0.05$. Except for twitch tension, the data were normally distributed with homogeneity of variance and thus were tested using one-way ANOVA and LSD-*t* (least significant difference) test of multiple comparisons. For the data

of twitch tension, the nonparametric Kruskal-Wallis test was used, combined with Dunnett-*t*-test (2-sided) for multiple comparisons between controls and treated groups.

RESULTS

Previous studies have shown that IGF1 and CT1 can increase EOM strength in adult and embryonic animals. Here we show that trophic factors can increase EOM strength in a juvenile maturing system.

Twitch Tension

IGF1 at a dose of either 5 μg (high dose) or 0.5 μg (low dose), CT1 at a dose of 5 μg, and combination of IGF1 0.5 μg and CT1 0.5 μg or 5 μg increased the maximum force produced in response to a single-pulse of direct trochlear nerve stimulation (15 V; Fig. 1A). The twitch tension of IGF1 (0.5 μg)-treated superior oblique was 7.11 $\pm$ 0.29 mN (mean $\pm$ SEM). Compared with PBS control (5.93 $\pm$ 0.30 mN) or normal control (6.21 $\pm$ 1.02 mN), the twitch tension increased by 19.9% and 14.5%, respectively ($P < 0.05$). The twitch tension of IGF1 (5 μg)-treated superior oblique was 7.00 $\pm$ 0.22 mN, with 12.7% to 18.0% increase in force. CT1 (5 μg) treatment, 7.41 $\pm$ 0.61 mN in muscle twitch tension, increased the tension 22.5% ($P < 0.05$), whereas CT1 0.5 μg did not increase the force (5.76 $\pm$ 0.26 mN) compared with controls. With combination treatment of IGF1 0.5 μg and CT1 5 μg, twitch tension was 7.31 $\pm$ 0.68 mN, a 20.8% increase in force ($P < 0.05$). IGF1 0.5 μg and CT1 0.5 μg combined treatment increased tension by 15.8%, to 7.00 $\pm$ 0.63 mN (Fig. 2A). Therefore, both doses of IGF1, 5 μg CT1, and combination treatment strengthened the single-twitch contractile force of the EOMs.

Tetanic Tension

IGF1 and CT1 treatment of juvenile EOMs of chickens increased tetanic tension (Fig. 1B) with a pattern and magnitude similar to those of twitch tension. Tetanic tension was signifi-
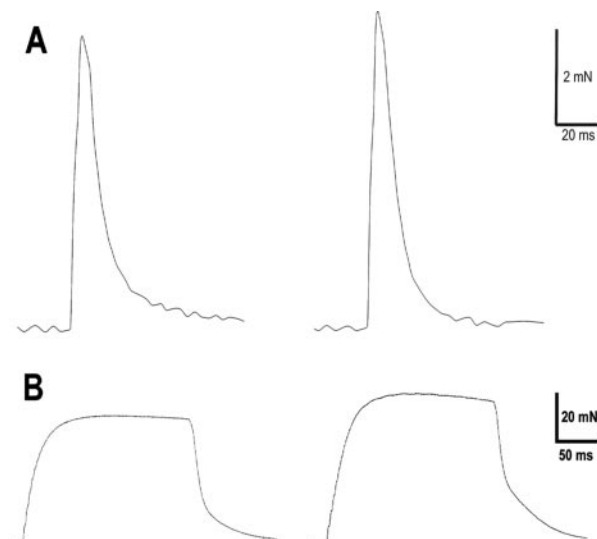
FIGURE 1. Tracing of twitch and tetanic tension of superior oblique muscles from 14-day-old chickens elicited by stimulation of the trochlear nerve. (**A**) Tracings of isometric twitch tension elicited by a single pulse (15-V, 0.2-ms duration) of a control muscle (*left*) and an IGF1 (0.5 μg)-treated muscle (*right*). (**B**) Tracing of tetanic tension elicited by stimulation with a train of pulses (15-V, 0.2-ms pulse duration, 450-Hz frequency, 200-ms train duration) of a control muscle (*left*) and an IGF1 (0.5 μg)-treated muscle (*right*).
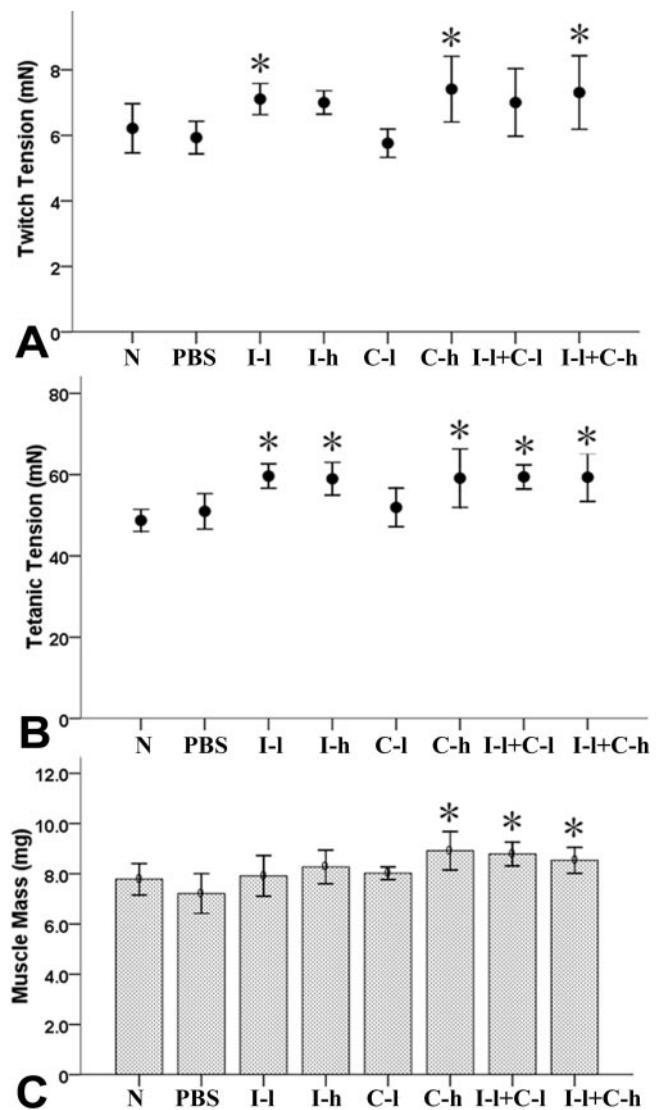
FIGURE 2. Quantification and comparison of three muscle parameters, twitch tension, tetanic tension, and muscle mass, for eight different conditions of the superior oblique muscle in 14-day-old chickens. Conditions include normal (N), injection of phosphate-buffered saline (PBS), injection of low-dose (0.5 μg) IGF1 (I-l), high-dose (5 μg) IGF1 (I-h), low-dose (0.5 μg) CT1 (C-l), high-dose (5 μg) CT1 (C-h), combination of low-dose IGF1 plus low-dose CT1 (I-l+C-l), and combination of low-dose IGF1 plus high-dose CT1 (I-l+C-h). Error bars, SEM; $n =$ 5–10 animals per data point. *Significant difference ($P < 0.05$) compared with controls. There was no significant difference between normal and PBS-injected muscles. (**A**) All trophic factors except for low-dose CT1 increased twitch tension, with low-dose IGF1, high-dose CT1, and low-dose IGF1 plus high-dose CT1 being statistically significant compared with PBS controls. (**B**) All treatment regimens with trophic factors except for low-dose CT1 increased maximal tetanic tension significantly. (**C**) High-dose CT1 and IGF1 plus CT1 combination treatment significantly increased muscle mass compared with PBS control.

cantly increased by the treatments of IGF1, CT1, and combination treatment. Tetanic tension of IGF1-treated muscles was 59.65 $\pm$ 1.83 mN (low dose) and 58.98 $\pm$ 2.45 mN (high dose). Compared with 48.73 $\pm$ 1.65 mN in normal controls and 50.97 $\pm$ 2.65 mN in PBS controls, IGF1 treatment increased tetanic tension by 17.0% to 22.4% (low dose, $P < 0.05$) and 15.7% to 21.0% (high dose, $P < 0.05$). Tetanic tension of CT1-treated muscles was 51.93 $\pm$ 2.88 mN (low dose) and
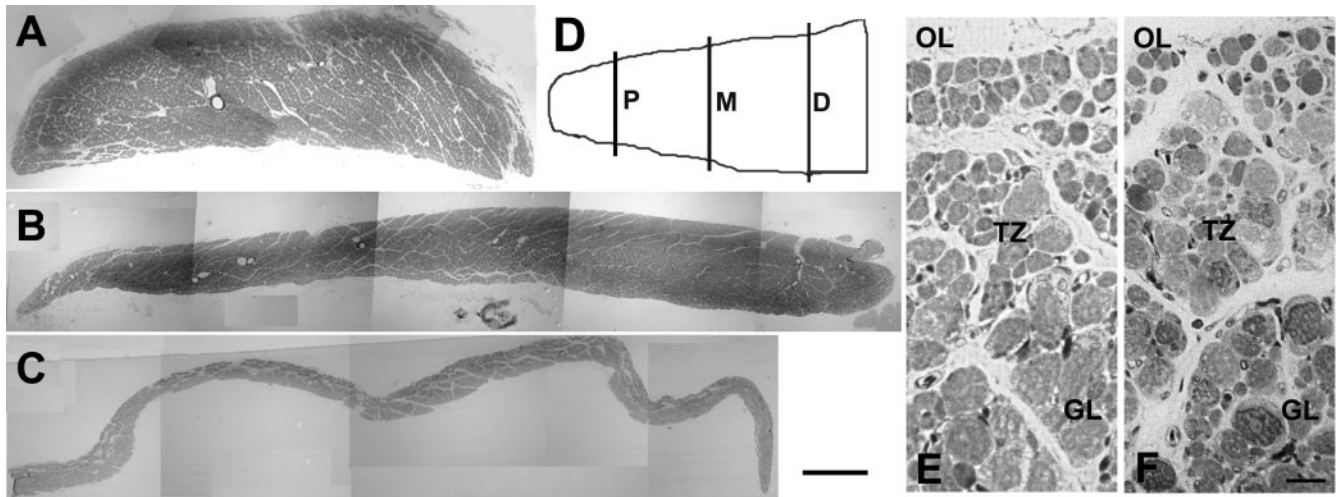
FIGURE 3. Representative examples of the histology of transverse sections through the normal superior oblique muscle of 14-day-old chickens at three levels: (**A**) proximal segment, (**B**) middle segment, (**C**) distal segment (attached to the eyeball). Semithin sections (1 μm) from resin-embedded muscles were stained with 0.7% toluidine blue. (**D**) Approximate levels of transverse sections in a schematic drawing of the muscle (P, proximal; M, middle; D, distal). In the normal muscle, the middle segment had the largest cross-sectional and myofiber area, whereas the distal segment had the smallest (see Fig. 4A). (**E**) Example of the orbital-global layer transition zone from a muscle stimulated at tetanic fusion frequencies. (**F**) Example of the orbital-global layer transition zone from an unstimulated muscle. OL, orbital layer; TZ, transition zone; GL, global layer. Scale bars: 100 μm (**A–C**); 10 μm (**E**, **F**).

59.13 $\pm$ 4.38 mN (high dose). CT1 treatment increased tetanic tension by 6.6% (low dose) and 18.2% (high dose, $P < 0.05$). Tetanic tension of muscles treated by IGF1 0.5 μg combined with CT1 0.5 μg or 5 μg was 59.43 $\pm$ 1.81 mN (IGF1 0.5 μg and CT1 0.5 μg) and 59.34 $\pm$ 3.60 mN (IGF1 0.5 μg and CT1 5 μg). Those treatments increased tetanic tension by 16.6% to 22.0% ($P < 0.05$) and 16.4% to 21.7% ($P < 0.05$; Fig. 2B). There was no increase in force with treatment when force was expressed as force/myofiber area or cross-sectional area. Fusion frequency (the lowest stimulation frequency at which individual twitches could not be differentiated at the tension plateau) for treated and control muscles was between 350 and 500 Hz. Such fusion frequencies are higher than in adult pigeon (190–250 Hz[28]), but they overlap or are within ranges previously reported for chicken (150–400 Hz[6,23]) and mammalian extraocular muscles (150–500 Hz; see Ref. 28 for review) and were below those for superfast avian skeletal muscles (600–800 Hz[29]). The treatment did not significantly change fusion frequency compared with controls. Maximum tetanic tension was reached at or close to the fusion frequency, and further increases of the rate of stimulation did not lead to an increase in the developed tension. Therefore, IGF1, CT1 (5 μg), and combination treatment increased the tetanic contraction of EOMs.

Effect of CT1 and IGF1 on EOM Mass

Muscle mass and muscle contractile force are correlated during chicken EOM development and maturation.[6] To determine whether muscle mass increases in a linear fashion with trophic factor-increased muscle force, we measured the mass of 54 superior oblique muscles of juvenile chickens after force measurement in situ. Stimulated and unstimulated EOMs showed no significant differences in mass, indicating lack of any significant edema. The mass of CT1 (5 μg)-treated muscles was 8.92 $\pm$ 0.47 mg. Compared with 7.78 $\pm$ 0.38 mg in normal chickens and 7.21 $\pm$ 0.48 mg in PBS controls, CT1 (5 μg) treatment increased the mass by 14.7% to 23.7% ($P < 0.05$). The mass of the muscles treated by IGF1 0.5 μg plus CT1 0.5 μg was 8.79 $\pm$ 0.29 mg, a 13.0% to 21.9% increase ($P < 0.05$). The mass of the muscles treated by IGF1 (0.5 μg) plus CT1 (5 μg) was 8.53 $\pm$ 0.31 mg compared with controls, a 9.6% to 18.3% increase ($P < 0.05$). Compared with PBS controls, the mass of IGF1-treated muscles was 7.91 $\pm$ 0.32 mg (low dose) and 8.27 $\pm$ 0.41 mg (high dose), which represent 9.7% and 14.7% increases, respectively; neither was significant with $P > 0.05$. CT1 (0.5 μg) treatment increased the mass by 11.2%, to 8.02 $\pm$ 0.15 mg (Fig. 2C). Therefore, the changes in contractile force induced by IGF1 and CT1 treatment were largely linear with increases in muscle mass, suggesting that the mechanism of force increase involves changes in muscle mass. This is consistent with the lack of significant differences between treated and control muscles when muscle force was normalized to the myofiber area.

Effect of CT1 and IGF1 on EOM Morphology

Muscle mass can be increased by increasing the diameter of individual myofibers (hypertrophy) or by increasing overall myofiber number (hyperplasia). Because myofibers are not continuous from the proximal to the distal insertion and because myofiber lengths differ between EOM segments[30] and myofiber types,[31] changes in muscle mass or morphology may differ along the longitudinal axis of EOMs. Therefore, we examined three segments (proximal, middle, and distal; Fig. 3) from superior oblique muscles of 14-day-old chickens that had been treated with CT1 (5 μg) or IGF1 (5 μg), using the same injection protocols as for force measurement. We chose the overall average muscle fiber diameter and fiber numbers in transverse sections as the parameters for the morphologic analysis.

Myofiber Area in Transverse Sections

In normal muscles, the myofiber area (as defined in Materials and Methods) of the proximal segment (0.95 $\pm$ 0.07 mm^2, attached on the interorbital septum) and the middle segment (1.16 $\pm$ 0.17 mm^2) was larger than that of the distal segment (0.55 $\pm$ 0.09 mm^2, attached on the sclera; Figs. 3, 4). The mean myofiber area was not significantly changed in the trophic factor-treated groups in the middle (IGF1, 1.28 $\pm$ 0.24 mm^2; CT1, 1.32 $\pm$ 0.05 mm^2) and distal segments (IGF1, 0.49 $\pm$ 0.13
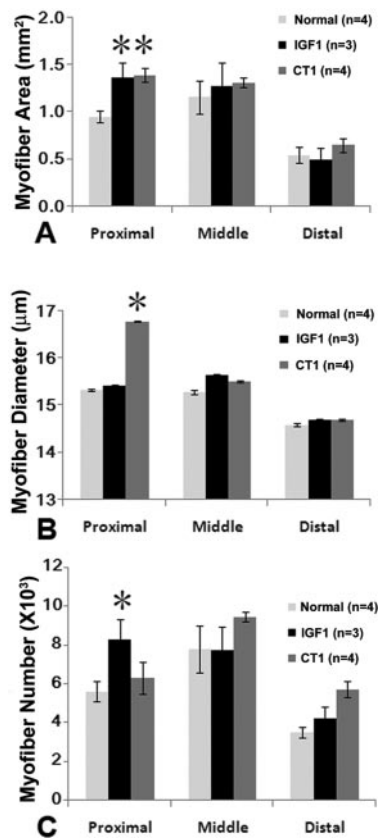
FIGURE 4. Quantification of three muscle parameters: average myofiber area, average myofiber diameter, and average myofiber number in transverse sections from three segments (proximal, middle, distal) in normal and IGF1- or CT1-treated superior oblique muscle. (**A**) IGF1 and CT1 treatment significantly increased myofiber area in the proximal segment. (**B**) CT1 treatment increased myofiber diameter in the proximal segment. (**C**) IGF1 treatment significantly increased myofiber number in the proximal segment, and CT1 treatment showed a trend toward increased myofiber numbers in the middle and distal segments. Error bars, SEM; $n = 3$–4. *Statistically significant difference compared with normal ($P < 0.05$).

mm^2; CT1, 0.65 ± 0.07 mm^2). In proximal segments, however, the myofiber areas of IGF1- and CT1-treated groups were increased significantly (IGF1, 1.36 ± 0.16 mm^2; CT1, 1.39 ± 0.07 mm^2) by approximately 40% and with a similar pattern compared with those of the normal proximal segment (0.95 ± 0.07 mm^2; Fig. 4A). The proximal segment constituted 35% to 40%, the middle segment 40% to 45%, and the distal segment 20% to 25% of the total myofiber area and, therefore, the contractile EOM mass.

Fiber Diameter

Increased myofiber area may be attributable to increased diameters of individual fibers or increased overall fiber numbers. To determine which parameter contributed to increased myofiber area after the IGF1 and CT1 treatment, we measured myofiber diameters. IGF1 and CT1 treatment did not significantly increase the fiber diameters of middle segments (15.65 ± 0.02 μm and 15.50 ± 0.02 μm, respectively). In distal segments of trophic factor–treated muscles, the fiber diameters also did not increase (14.58 ± 0.04 μm vs. 14.69 ± 0.02 μm). However, CT1 treatment significantly increased the fiber diameter in the proximal segment of treated muscles (16.78 ± 0.02 μm), by >8% ($P < 0.05$), compared with that (15.28 ± 0.05 μm) in the normal proximal segment (Fig. 4B). Given that area = πr^2, the

increase in myofiber diameter accounts for more than half (20% of the 40%) of the increase in myofiber area seen with trophic factor treatment. To determine how CT1 and IGF1 altered the distribution pattern of myofiber diameter, we analyzed the relative frequency of myofiber diameters in histograms. We found that IGF1 and CT1 both shifted the fraction of global myofibers to larger diameters. IGF1 also increased the fraction of smaller diameter fibers (mostly orbital and small global fibers), which may represent the new fibers (Fig. 5).

Fiber Numbers in Transverse Sections

In addition to myofiber diameter, myofiber number may contribute to increased myofiber area. To determine whether the muscle may exhibit hyperplasia or increased myofiber length (each of which is difficult to distinguish in EOMs), we quantified the myofiber number in transverse sections. IGF1 treatment significantly increased myofiber number in the proximal segment from 5626 ± 522 to 8323 ± 1016. CT1 treatment increased fiber number (9479 ± 254) of the middle segment, but without statistical significance, compared with that (7762 ± 1209) of the normal middle segment. Between control and treated groups, the fiber number of distal segments did not show any significant change (Fig. 4C). The considerable increase in myofiber area with IGF1 and CT1 treatment was attributed to the remodeling of proximal segments, and this change was caused by increased myofiber diameters by CT1 but mostly by increased myofiber number or length by IGF1.

Accumulation of IGF1 and CT1 in EOMs

To exclude the possibility that differences in trophic factor availability or access between EOM segments caused by technical aspects of injections may explain segmental differences in effects, we injected 0.5 μg radiolabeled trophic factor and γ-counted eight EOMs separately for each of the three segments (proximal, middle, distal). There was no significant difference in trophic factor accumulation between segments, based either on total cpm or cpm/EOM mass.

Activated Satellite Cells Labeled by BrdU

The above data indicate that muscle changes associated with strengthening are located in proximal, rather than distal, segments of EOMs. To determine whether this asymmetry is reflected by satellite cell proliferation, we asked whether IGF1 and CT1 induced the proliferation of satellite cells and how the myonuclear addition was distributed throughout the EOM. We identified proliferating satellite cells and integrated nuclei using BrdU immunolabeling. In longitudinal sections, the BrdU-positive nuclei were solid and oval and were located typically on the periphery of myofibers (Fig. 6A). In the normal control muscles, BrdU-labeled satellite cells had a similar distribution from proximal to distal segments (proximal 0.50 ± 0.06, middle 0.42 ± 0.05, and distal 0.56 ± 0.07 labeled nuclei per 100 μm myofiber). IGF1 and CT1 treatment significantly increased numbers of BrdU-positive nuclei in the proximal and middle segments. The proximal segment had the largest increase in BrdU-labeled cells (0.78 ± 0.15 nuclei in CT1-treated muscles and 0.85 ± 0.18 nuclei in IGF1-treated muscles for each 100 μm myofiber); the middle segment also had significant increases in BrdU-positive nuclei (0.56 ± 0.10 for CT1 and 0.61 ± 0.07 for IGF1); the distal segment did not show any significant increase in satellite cell proliferation after IGF1 and CT1 treatment (Fig. 6B). Therefore, proliferating satellite cells were primarily, but not exclusively, increased in muscle segments that showed significant muscle remodeling after IGF1 and CT1 treatment.
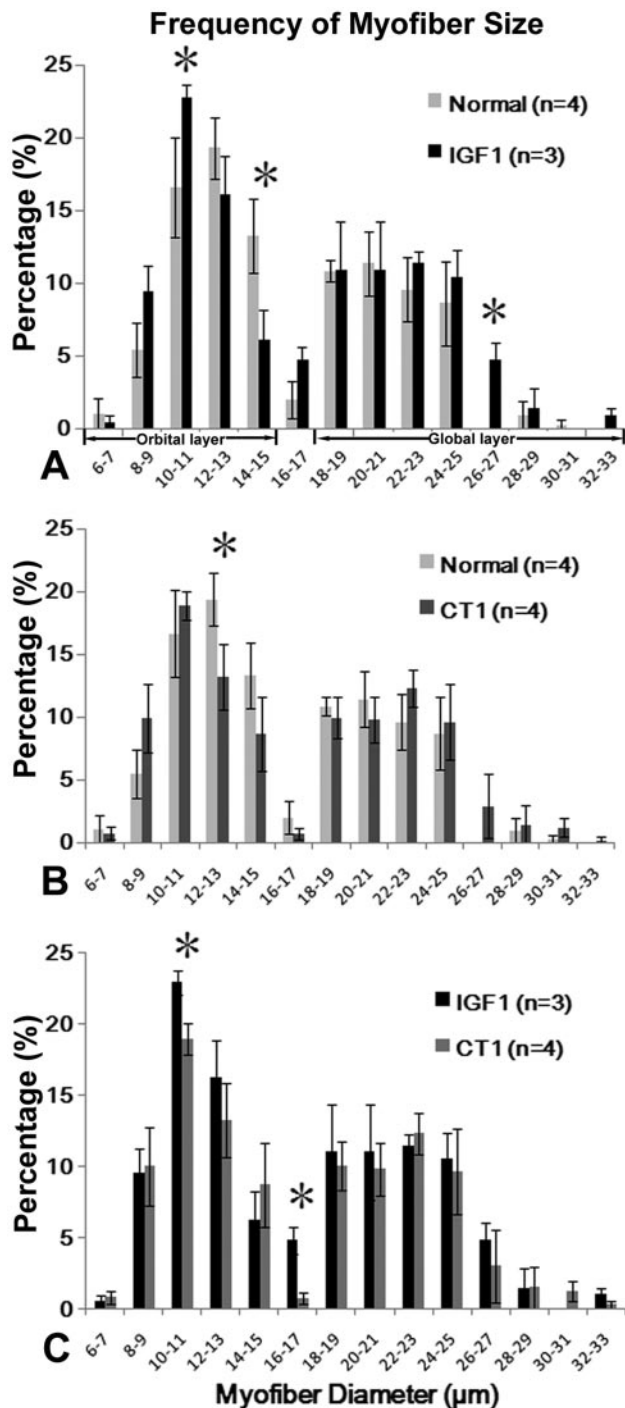
FIGURE 5. Comparison of the distribution of myofiber diameters in proximal segments of normal and IGF1-treated (**A**) or normal and CT1-treated (**B**) or comparison between IGF1- and CT1-treated superior oblique muscles of 14-day-old chickens (**C**). The first peak (6–15 μm) corresponds largely with orbital layer fibers, and the second peak (17–33 μm) with global layer fibers. (**A**) IGF1 increases the percentage of large diameter fibers but also shows larger numbers of smaller myofibers (possibly new or elongated fibers; see Fig. 4C). (**B**) CT1 increases the percentage of the larger myofibers (diameters > 22 μm) more than the percentage of the smaller myofibers (diameters = 8–11 μm), thus resulting in a net increase in the average fiber diameter (Fig. 4B). (**C**) Direct comparison of IGF1 and CT1 shows a very similar effect, with minimal differences in percentages of fiber diameters. Error bars, SEM; n = 3–4. *Statistically significant difference ($P <$ 0.05).

DISCUSSION

Maturing EOMs Respond to Trophic Factors

It is well known that strabismus should be corrected in infants to allow the development of normal neurovisual circuits for binocular vision; otherwise, strabismus can cause severe deficiencies such as amblyopia. Trophic factors are promising candidates for strengthening underacting EOMs to treat strabismus.[3,5] To explore the plasticity of maturing EOMs, we chose the maturing chicken (P7-P14) as our animal model. Previous research had not examined the effects of trophic factors on juvenile EOMs or any synergism between trophic factors. IGF1 and CT1 use, at least in part, different signal transduction pathways: IGF1 activates the calcineurin pathway in association with GATA-2 and NF-ATc1[32] and the PtdIns-3-OH kinase in association with glycogen synthase kinase 3,[33] whereas CT1 activates the JAK-STAT pathway cascade. Therefore, we explored the possibility of additive effects, even though both trophic factors are known to induce the proliferation of satellite cells in skeletal muscle.[20,21,34] We show that both IGF1 and CT1 increased contractile force, myofiber area, and number of proliferating satellite cells of EOMs in juvenile chickens, but there was no additive effect of the two trophic factors on either contractile force or morphologic muscle parameters. These data show that maturing EOMs respond to both trophic factors, though apparently with different mechanisms of action.

Regional Differences in Alteration of EOMs

Previous work has shown that muscle parameters within EOMs can differ substantially between different regions of the muscle. For example, the myofiber number and myosin heavy chain isoform composition differ between proximal and distal segments of adult mammalian EOMs.[30,35] Therefore, we examined whether different segments of the maturing chicken EOMs may respond differently to the trophic factors IGF1 and CT1. Interestingly, we found that morphologic changes within the proximal segment of the EOMs appear to be sufficient to implement substantial increases in the contractile force of the treated muscles (Fig. 4A). Even though the trophic factor–treated EOMs were approximately 20% stronger than their controls, the distal segment, and for most parameters the middle segment as well, showed no significant changes in myofiber diameter, number, or myofiber area. Compared with human EOMs, it would be interesting to know whether morphologic alterations in strabismic human EOMs may be restricted similarly to the proximal segment given that there appear to be only discrete ultrastructural changes in the distal segment of myofibers from strabismic EOMs, which is the segment that is typically accessible for examination after strabismus surgery in humans.[36]

Mechanism of Force Increase: Hypertrophy, Hyperplasia, and Muscle Lengthening

Previous studies on the anatomic correlates of increased contractile force in EOMs have resulted in partially conflicting reports. McLoon et al.[14] found overall unchanged mean myofiber diameters in adult rabbit EOMs with IGF2 treatment but increased diameters of the largest fibers and decreased diameters of the smallest fibers. However, when treated with IGF1 and CT1 in embryonic chicken, EOMs shifted the diameters of myofibers across the board to larger diameters, and function-blocking proteins of antibodies against these trophic factors reduced diameters in all fiber ranges.[15] Adult muscles with multiple endplate bands respond to hypertrophic stimulation by increasing individual myofiber length, which was shown by

FIGURE 6. (A) Example of bromode-oxyuridine (BrdU) immunolabeling in longitudinally sectioned myofibers of a CT1-treated superior oblique muscle from a 14-day-old chicken. (B) The average number of BrdU-positive nuclei per 100 μm myofiber from three segments (proximal, middle, distal). Both IGF1 (5 μg) and CT1 (5 μg) treatments significantly increased the number of BrdU-positive nuclei in proximal and middle segments. Error bars, SEM; n = 5–7. *Statistically significant difference ($P < 0.05$).
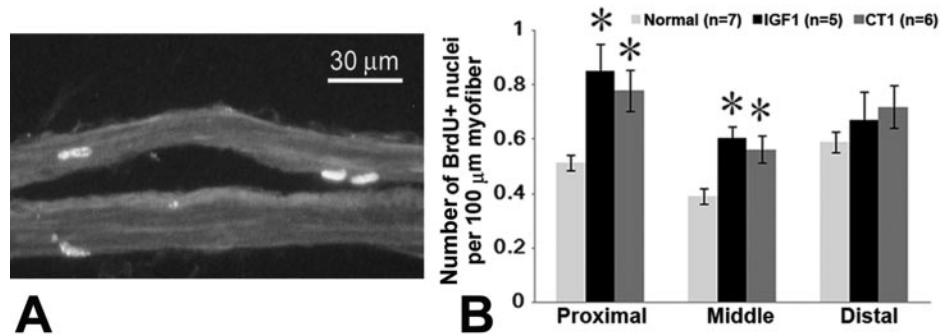
increased myofiber numbers in transverse sections but no increased fiber count after acid digestion of whole muscle.[37] Because the muscle mass appeared increased in adult EOMs even when the mean myofiber diameters were unchanged, it was suggested that the mechanism of force increase may involve muscle lengthening rather than myofiber hypertrophy.[14,37] In EOMs, many myofibers do not extend from the proximal to the distal site of insertion[30,31] and therefore have the potential to increase in length in response to changes in demand. In our study on juvenile EOMs, we found that, in the proximal segment, myofiber diameter but not number was increased with CT1, whereas for IGF1, myofiber number (or length) increased, but diameter increase in the global layer was accompanied by an increased number of smaller myofibers (Fig. 5). The increase in myofiber diameter with CT1 accounted for much of the increased myofiber area of the proximal EOM segment, whereas the increased myofiber number (or length) with IGF1 may largely account for the increased myofiber area. Given that the muscle mass is largely composed of the proximal and middle segments (75%–85%) and the distal segment contributes only 20–25% (see Results), a 40% change in myofiber area in the proximal segment may be responsible for a 20% increase in contractile force.

It is surprising that the different mechanisms for increases in myofiber area (and muscle mass) with IGF1 and CT1 (fiber number/length vs. hypertrophy, respectively) did not show any additive, synergistic effects in the combination treatment on contractile force because one factor could have theoretically increased fiber number and the other could have increased myofiber diameter, resulting in an even greater change. It is well established that, in the theory of muscle growth, additional sarcomeres or myofibrils generated in parallel cause contractile force enhancement.[38,39] From our morphologic analysis, it is not possible to conclude whether the trophic factors acted on the same or different myofiber populations. It should be noted that trophic factors may change additional parameters relevant for muscle strength, not examined here, such as changes in the distribution and size of mitochondria or the composition of myofiber types (Li T, et al. *IOVS* 2008;49:ARVO E-Abstract 4493).

Hypertrophy and Satellite Cell–Mediated Myonuclear Addition

The activity of satellite cells is essential for growth, regeneration, and adaptive functions of skeletal muscle. In the absence of disease or injury, the satellite cell activity of adult skeletal muscle is extremely low.[34] However, EOMs are unique in that normal mature EOMs contain activated satellite cells, indicating continuous myonuclear addition.[27,40] Therefore, they may respond to trophic factors in a fashion different from those in skeletal muscle. Furthermore, adult rabbit EOMs have a higher percentage of activated satellite cells in the distal tendon end region.[27] Consistent with this report, we found that the activity

of satellite cells in the distal segments had the highest level in normal superior oblique muscle. Surprisingly, however, the satellite cell activity in distal segments did not increase significantly in response to trophic factors, possibly because their activity was already at maximal levels. With IGF1 and CT1 treatment, the proximal segment of the chicken superior oblique muscle was remodeled to increase myofiber area, which indicates new protein synthesis (for larger fibers or new fibers), consistent with our finding that the proximal segment showed the most pronounced morphologic changes. Thus, important muscle parameters of plasticity in maturing EOMs can be manipulated by treatment with distinct trophic factors.

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